Calcium-dependent inactivation of heteromeric NMDA receptor–channels expressed in human embryonic kidney cells

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1. Whole-cell currents through heteromeric NR1–NR2A and NR1–NR2B subunit combinations of NMDA channels transiently expressed in human embryonic kidney cells (HEK 293) were studied using the patch-clamp technique.

2. With 4 mM Mg-ATP in the internal pipette solution, the responses of cells expressing NR1–NR2A channels to glutamate application gradually decreased, reaching 50% of control during the first 20 min of recording. This process was accompanied by acceleration of desensitization.

3. Conditioning (5–15 s) applications of glutamate (100 μM) induced a transient inactivation of NR1–NR2A and NR1–NR2B channels (20–40%) with a slow time course of recovery (τ_r = 10–60 s). Both the degree of inactivation and the time constant of recovery increased with the duration of conditioning applications of glutamate, and with an elevation of Ca^{2+} in the external solution.

4. These results show that both NR1–NR2A and NR1–NR2B recombinant NMDA receptor–channels expressed in HEK 293 cells can be transiently inhibited by Ca^{2+} ions in a similar way to that described for hippocampal neurones.

The NMDA subtype of glutamate receptors has a number of regulatory sites on the external and internal parts of the plasma membrane. It has been shown that intracellular calcium ($[Ca^{2+}]_i$) can transiently inhibit the activity of NMDA channels (Mayer & Westbrook, 1985; Zorumski, Yang & Fischbach, 1989; Legendre, Rosenmund & Westbrook, 1993; Medina, Bregestovski & Ben-Ari, 1993; Vyklický, 1993). This process (Ca^{2+}-dependent inactivation) has several specific features: maximal inhibition does not exceed 50%, recovery is relatively slow (10–50 s), and it is not modulated by ATP, phosphatase inhibitors or other regulators of phosphorylation (Legendre *et al.* 1993; Rosenmund & Westbrook, 1993*a*; Vyklický, 1993; Medina, Filippova, Barbin, Ben-Ari & Bregestovski, 1994). Results obtained at the single-channel level suggest that the cytoplasmic environment may be important for the Ca^{2+}-dependent inactivation of NMDA channel activity (Legendre *et al.* 1993). As one possible mechanism of NMDA channel regulation by $[Ca^{2+}]_i$ it has been suggested that a regulatory Ca^{2+}-dependent cytoskeleton protein is linked to the NMDA receptor (Rosenmund & Westbrook, 1993*b*). Recently, it has also been documented that $[Ca^{2+}]_i$ can modulate NMDA channels through activation of the calcium–calmodulin-dependent phosphatase calcineurin (Lieberman & Mody, 1994; Tong & Jahr, 1994).

To evaluate whether the Ca^{2+}-dependent inactivation of NMDA channels is an intrinsic property of the receptor–channel protein or is determined by the neurone-specific environment (lipid membrane or intracellular components), we have studied this phenomenon on NMDA receptor heteromeric subunits expressed in human embryonic cells (HEK 293).

METHODS

Cell culture and transfection

Insertions encoding the various NMDA receptor subunits (Monyer *et al.* 1992) were spliced out from their native Bluescript plasmids (Stratagene, La Jolla, CA, USA) and subcloned into the eukaryotic expression vector pcDNA I/Amp (Invitrogene, San Diego, CA, USA). Immortalized human embryonic kidney cells

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(HEK 293) were cultured on 35 mm tissue culture plates (5×10^5 to 10^6 cells per plate) in complete medium containing 10% calf serum. Cells were rinsed in serum-free medium and transfections were performed in reduced-serum medium (Opti-MEM; Gibco BRL, Gaithersburg, USA) containing 10 μl lipofectamine (Gibco BRL) mixed with an equal weight (1 μg) of subunit-specific cDNA. Following 5 h incubation, an equal volume of complete growth medium containing twice the concentration of serum was added to the transfection mixture. The next day, this medium was replaced with fresh complete medium and cells were used for recording 24–48 h later.

Electrophysiology

Petri dishes with transfected cells were transferred to the stage of an inverted microscope and bathed continuously with solution containing (mM): NaCl, 140; KCl, 3·5; glucose, 10; $CaCl_2$, 2·0; Na-Hepes, 10 (pH 7·4: osmolarity, 300 mosmol (l solution)$^{-1}$). The borosilicate glass pipettes had resistances of 2–7 MΩ when filled with the internal solution containing (mM): CsCl, 130; $CaCl_2$, 0·25; EGTA, 1·1; Hepes, 10; Mg-ATP, 4·0; creatine phosphate, 6·0 (pH 7·2; osmolarity, 280 mosmol (l solution)$^{-1}$). L-Glutamate (100 μM) and glycine (10 μM) dissolved in the extracellular solution were applied to the cells by either a fast-perfusion system or by pressure through a micropipette (tip diameter of 2 μm) using a 'Picospritzer' (General Valve Corporation, Fairfield, NJ, USA) as previously described (Medina *et al.* 1994). To study the Mg^{2+} block of NMDA currents an extracellular solution containing 0·5–1 mM Mg^{2+} was used.

Whole-cell currents were recorded from single cells at room temperature (19–22 °C) using an EPC-9 amplifier (Heka Electronics, Liambrecht, Germany) and stored on digital tape using a DTR 1201 recorder (Bio-Logic, Claix, France) for analysis. The holding potential was −50 mV in all experiments. Results of only those experiments in which the amplitude of glutamate-evoked currents was larger than 50 pA are presented.

For analysis, data were filtered at 1–3 kHz and digitized using an IBM IT computer (Intersys, Torrance, CA, USA) at 5–10 kHz using pCLAMP software (Axon Instruments, USA) and a Labmaster DMA board (DIPSI, Asnières, France). All results are presented as means ± S.E.M. Statistical comparisons were made using Student's *t* test.

RESULTS

Figure 1*A* and *C* shows typical whole-cell currents recorded from HEK 293 cells expressing NR1–NR2A or

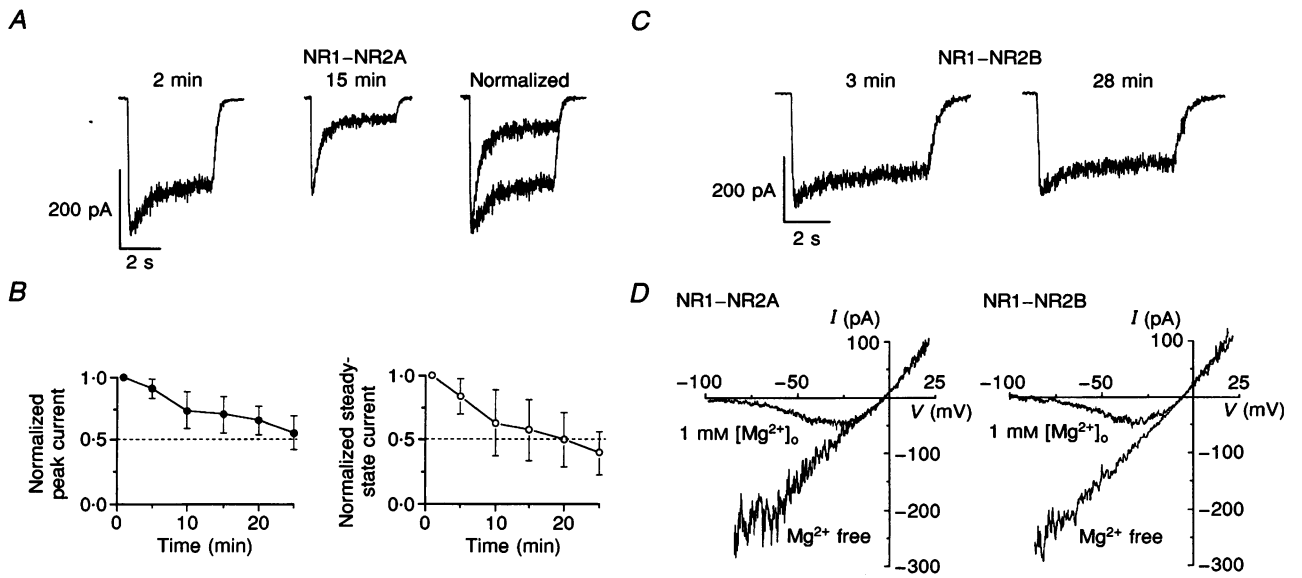

Figure 1. Properties of two types of recombinant NMDA receptor–channel

A, responses to simultaneous applications of 100 μM glutamate and 10 μM glycine through a fast-perfusion system to cells expressing the NR1–NR2A channel at different times during whole-cell recording (indicated above the traces). Time zero was taken as the start of the whole-cell recording. Note the gradual decrease of the steady-state component of the responses and the acceleration of the desensitization degree during recording. *B*, amplitudes of peak (●) and steady-state (○) currents plotted against the time of whole-cell recording. Data are normalized relative to the first response (40–60 s after breakthrough into whole-cell configuration) and plotted as means ± S.E.M. from a total of five patches. After 30 min of recording the currents had declined to 56 ± 13 and 40 ± 17% of the peak and steady-state amplitudes, respectively. *C*, responses to simultaneous applications of 100 μM glutamate and 10 μM glycine through a fast-perfusion system in cells expressing the NR1–NR2B channel at the third and twenty-eighth minute of whole-cell recording. *D*, whole-cell current–voltage relationships of responses to glutamate in cells expressing the NR1–NR2A channel (left panel) and the NR1–NR2B channel (right panel) in the absence of extracellular Mg^{2+}, and in the presence of 1 mM $[Mg^{2+}]_o$. Currents in the absence of agonist were subtracted.

NR1–NR2B subunit combinations. Fast application of glutamate (100 μM in 5 s) in a solution containing 10 μM glycine evoked whole-cell currents with amplitudes from 30 to 600 pA in different cells. The offset decay time constants (see Monyer *et al.* 1992) measured at a holding potential of −50 mV in Mg^{2+}-free solution were 137 ± 11 ($n = 7$) and 350 ± 25 ms ($n = 4$) for NR1–NR2A and NR1–NR2B channels, respectively. With 0·5–1 mM Mg^{2+} in the external solution, current–voltage (I–V) relationships of glutamate responses were similar for both recombinant channels in the negative membrane potential range, with maximum inward currents around −30 mV. In Mg^{2+}-free solution, the I–V relationships were linear (Fig. 1*D*). Thus, the main properties of both recombinant NR1–NR2A and NR1–NR2B channels in our experiments were similar to those described previously (Monyer *et al.* 1992; Monyer, Burnashev, Laurie, Sakmann & Seeburg, 1994).

During the application of long (2–5 s) pulses of glutamate to cells expressing NR1–NR2A or NR1–NR2B channels, inward currents slowly declined ('desensitized') (Figs 1*A* and *C*, 2*A*, 3*A* and 4*A*). At the beginning of whole-cell recording, in cells expressing the NR1–NR2A subunit combination, the degree of desensitization was variable. For one group of cells, glutamate currents declined in a single slow phase which, on average, had a time constant ($\tau_{on,s}$) of 3950 ± 300 ms ($n = 8$) during the third minute of recording. In other cells, the glutamate-induced currents declined from the beginning with kinetics which could be fitted by two exponentials (fast and slow). On average, $\tau_{on,f}$ was 558 ± 101 ms and $\tau_{on,s}$ was 3100 ± 770 ms

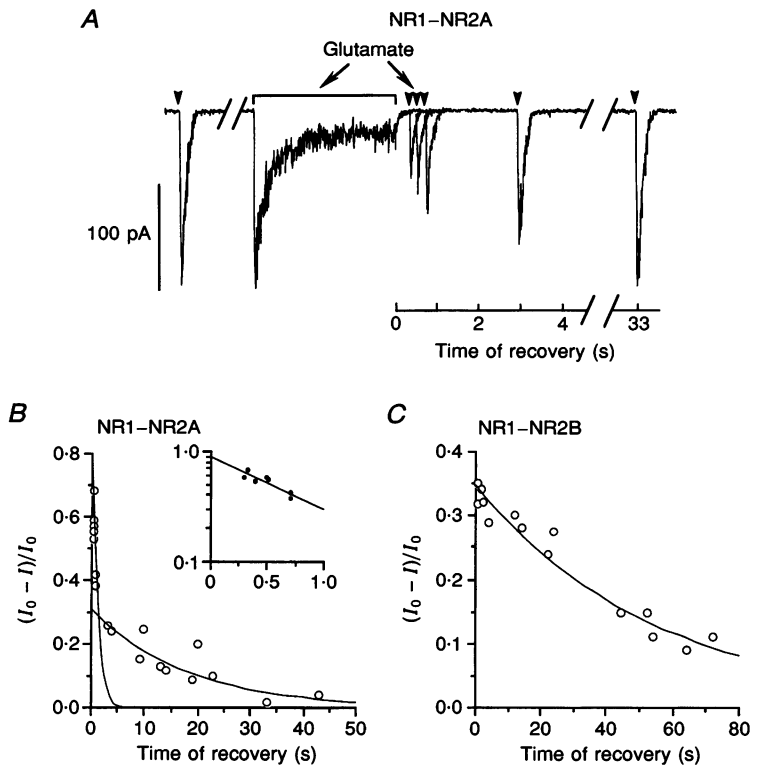

Figure 2. Time course of NR1–NR2A and NR1–NR2B channel recovery from glutamate-induced inactivation

A, illustration of the double-pulse experiment. Inward currents were generated by coapplications of 100 μM glutamate and 10 μM glycine to a cell expressing the NR1–NR2A channel. Long conditioning applications were made using a fast-perfusion system. Short (50 ms) pulses of glutamate were applied through a micropipette by pressure every 10 s and at various times after the end of the conditioning application (as indicated by the bar and arrowheads above the current records). The traces of the test responses are superimposed. *B*, time course of the recovery of test currents in a cell expressing the NR1–NR2A channel. Data from eight measurements are plotted. I_0 and I are the amplitudes of the test pulses before and after the conditioning pulse, respectively. The recovery was fitted by a double exponential, with time constants of 1·4 (see inset) and 18 s. Inset: semilogarithmic plot of the time course of the fast component of recovery. *C*, time course of test current recovery in a cell expressing the NR1–NR2B channel. The recovery was approximated by a single exponential with a time constant of 54 s.

($n = 5$). Within 5–15 min of the whole-cell dialysis the fast component of desensitization appeared in all cells expressing the NR1–NR2A channel and, after 20 min, the time course of desensitization could be fitted by two exponentials with a $\tau_{on,f}$ of 364 ± 53 ms and a $\tau_{on,s}$ of 2176 ± 293 ms ($n = 13$).

In general, during long-lasting recordings (20–30 min) from cells expressing the NR1–NR2A channel, two main phenomena were observed: (i) the amplitude of the glutamate responses decreased gradually, and (ii) the speed of desensitization was accelerated progressively (Fig. 1*A*). As a result, the amplitude of the plateau component declined faster than the amplitude of the peak (Fig. 1*B*).

To study Ca^{2+}-induced inactivation of NMDA channels expressed in HEK 293 cells we used a double-pulse experimental protocol (Fig. 2*A*), similar to that reported in a previous study (Medina *et al.* 1994). Brief (50 ms) test pulses of glutamate applied repeatedly at intervals of 10 s generated highly reproducible inward currents with peak amplitudes between 50 and 300 pA. In cells expressing NR1–NR2A or NR1–NR2B channels, application of conditioning (2–20 s) pulses of glutamate caused a strong suppression of the glutamate test currents (Figs 2*B* and *C*, 3*A* and *C*, and 4*A*). By varying the interval between the end of the conditioning pulse and the beginning of the test pulse (see Fig. 2*A*), we estimated the recovery time constant of glutamate-induced test currents.

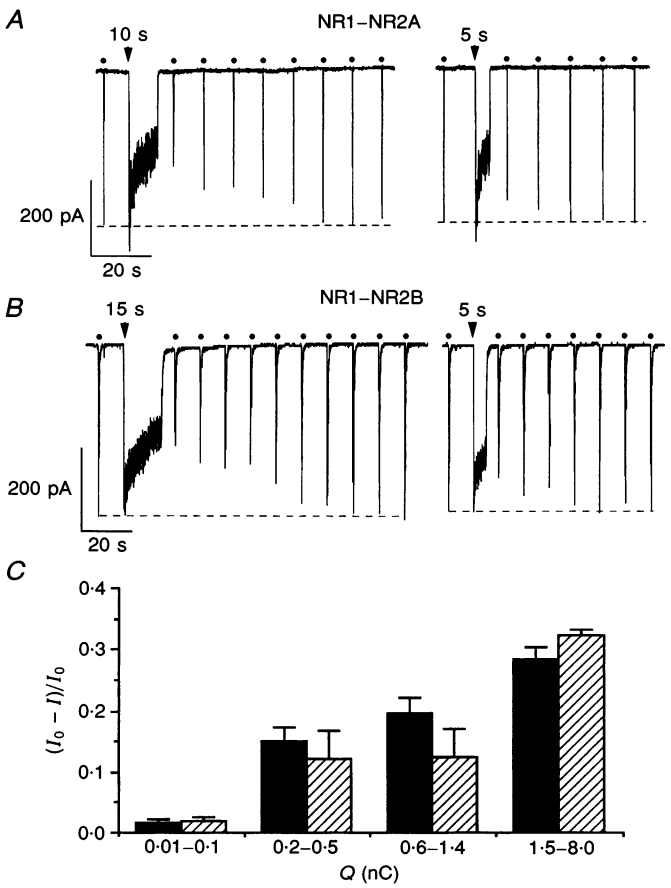

Figure 3. The degree of inactivation and the recovery time course of recombinant NR1–NR2 channels during different conditioning glutamate applications

A and *B*, whole-cell currents evoked by brief (50 ms) test (●) and long (2–15 s) conditioning (▼) pressure applications of 100 μM glutamate and 10 μM glycine to a cell expressing the NR1–NR2A channel (*A*) and the NR1–NR2B channel (*B*). The degree of inactivation was calculated as $(I_0 - I)/I_0$, where I_0 is the average amplitude of 3–4 test currents before conditioning applications of agonists, I is the amplitude of the test current measured 4–5 s after the end of the conditioning application (to avoid influence of the fast component of recovery, observed for NR1–NR2A channel (see Fig. 2*B*)). *C*, dependence of the degree of inactivation observed for the NR1–NR2A (■) and NR1–NR2B (▨) channel on the charge (Q) passed through channels during conditioning applications of glutamate. The columns represent the mean degree of inactivation ± S.E.M. calculated for the different ranges of charge indicated below the columns.

For the NR1–NR2A subunit combination, the recovery of glutamate-induced test currents developed in two distinct phases (Fig. 2*B*). The time constant of the first phase had a mean value of 1·3 ± 0·14 s ($n = 6$). The time constant for the second component ($\tau_{off,s}$) was in the range 10–60 s. A similar two-phase recovery was observed in thirteen of sixteen cells expressing the NR1–NR2A channel, whereas in the other three cells only the fast component was observed. In contrast, in four of five cells expressing the NR1–NR2B channel, the recovery developed in a single slow phase (Fig. 2*C*) and in one cell (current amplitude of 210 pA), inhibition of the test current by conditioning pulses of glutamate was absent.

In a previous study on hippocampal neurones (Medina *et al.* 1994) we have shown that this slow component reflects the recovery of NMDA channels from Ca^{2+}-induced inactivation. Results summarized in Figs 3 and 4 show that in recombinantly expressed NMDA receptor–channels this phenomenon is also Ca^{2+} dependent and the main properties of the slow component of the recovery are similar to those obtained in hippocampal neurones.

For both NR1–NR2A and NR1–NR2B channels, the degree of glutamate test-current inhibition and the time course of the recovery were proportional to the duration of the conditioning glutamate pulse. For example, for the NR1–NR2A channel on the cell depicted in Fig. 3*A*, the rates of inactivation were 29 and 16% and the time constants of the recovery were 19 and 11 s for 10 and 5 s conditioning pulses, respectively. For the NR1–NR2B channel (Fig. 3*B*), the rates of inactivation were 32 and 19% and the time constants of the recovery were 36 and 24 s for 15 and 5 s conditioning pulses, respectively.

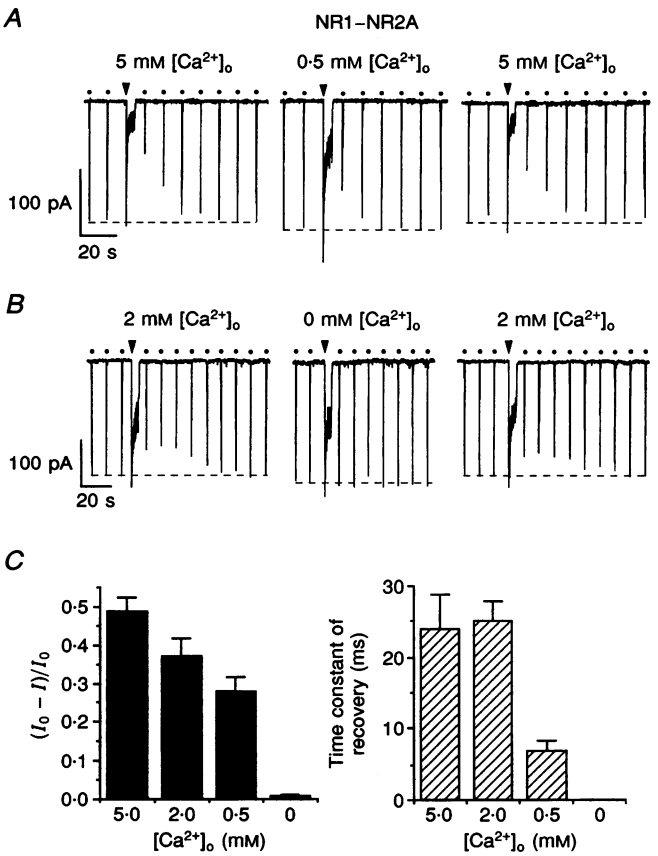

Figure 4. Effect of $[Ca^{2+}]_o$ on the degree of inactivation and the recovery time course of the NR1–NR2A channel

A, glutamate-activated currents in 5 and 0·5 mM $[Ca^{2+}]_o$. Experimental protocol as described in Fig. 3; duration of the conditioning pulse was 5 s. The application of 100 μM glutamate and 10 μM glycine were performed using two different micropipettes containing 0·5 (middle trace) and 5 mM $[Ca^{2+}]_o$ (left and right traces). At least 1 min prior to application of the corresponding conditioning pulse, cells were bathed with solutions containing 5 or 0·5 mM $[Ca^{2+}]_o$, respectively. The degree of inactivation was 56, 31 and 44% for 5, 0·5 and (again) 5 mM $[Ca^{2+}]_o$, respectively. *B*, glutamate-activated currents in control (2 mM $[Ca^{2+}]_o$) and in nominal absence of Ca^{2+} in the external solution. Experimental protocol is as described above. *C*, dependence of the degree of inactivation and the time constant of recovery on $[Ca^{2+}]_o$. Mean data from 3 to 5 cells are presented.

To compare results from different experiments on cells expressing NR1–NR2A and NR1–NR2B channels, we estimated the dependence of the degree of inhibition ($(I_o - I)/I_o$) on the charge (Q) passing through the cell at the application of conditioning pulses: $Q = It$ (in nanocoulombs), where I is the amplitude of glutamate-induced current and t is the duration of conditioning pulse. For both recombinant channels the dependence of the degree of inactivation on the amount of charge passing through the cell was similar (Fig. 3*C*). At 2 mM $[Ca^{2+}]_o$ the thresholds of inhibition were in the range of $Q = 0{\cdot}01$–$0{\cdot}1$ nC; with Q of 0·2–0·5 nC the degrees of inhibition were $0{\cdot}15 \pm 0{\cdot}02$ ($n = 17$) and $0{\cdot}12 \pm 0{\cdot}05$ ($n = 5$) for NR1–NR2A and NR1–NR2B channels, respectively. The difference was not significant ($P > 0{\cdot}05$). The maximal degrees of inactivation were obtained when Q measured 1·5–2·0 nC; on average, $(I_o - I)/I_o$ values were $0{\cdot}28 \pm 0{\cdot}02$ ($n = 6$) and $0{\cdot}32 \pm 0{\cdot}01$ ($n = 3$) for NR1–NR2A and NR1–NR2B channels, respectively. The difference was not significant ($P > 0{\cdot}05$). These data suggest that the properties of Ca^{2+}-induced inactivation are similar for NR1–NR2A and NR1–NR2B channels.

To demonstrate directly that this type of inhibition is evoked by an elevation of $[Ca^{2+}]_i$, we analysed the degree of NR1–NR2A channel inactivation in different concentrations of external Ca^{2+}. Figure 4*A* illustrates that a 10-fold reduction of $[Ca^{2+}]_o$ reversibly decreased the inhibitory effect of conditioning glutamate and accelerated the time course of the recovery. In the presence of 5 mM $[Ca^{2+}]_o$ the amplitude of the glutamate-induced current was smaller than in 0·5 mM $[Ca^{2+}]_o$. Accordingly, the amount of charge passing through the cell with application of the conditioning pulses in 5 mM $[Ca^{2+}]_o$ was 2·6-fold less than that in 0·5 mM $[Ca^{2+}]_o$. On average (Fig. 4*C*) the degree of inactivation was $0{\cdot}49 \pm 0{\cdot}04$ ($n = 4$) in 5 mM $[Ca^{2+}]_o$ and $0{\cdot}28 \pm 0{\cdot}035$ ($n = 4$) in 0·5 mM $[Ca^{2+}]_o$. The difference was significant, $P < 0{\cdot}05$. Time constants of the recovery from inactivation were similar in both 5 and 2 mM $[Ca^{2+}]_o$ ($24 \pm 4{\cdot}9$ s, $n = 4$ and $25 \pm 3{\cdot}0$ s, $n = 6$, respectively) and decreased significantly ($P < 0{\cdot}05$) in 0·5 mM $[Ca^{2+}]_o$ ($9 \pm 1{\cdot}8$ s, $n = 4$) (Fig. 4*C*). When Ca^{2+} was removed from the external solution (Fig. 4*B* and *C*) a long conditioning application of glutamate did not induce inactivation ($n = 6$).

DISCUSSION

The main finding of this study is that the activity of NR1–NR2A and NR1–NR2B channels, expressed in HEK 293 cells, can be transiently inhibited by elevation of $[Ca^{2+}]_i$. As the main source for $[Ca^{2+}]_i$ elevation we used the NMDA channels themselves, which are highly permeable to Ca^{2+} ions (Mayer & Westbrook, 1987; Schneggenburger, Zhou, Konnerth & Neher, 1993).

Properties of the inactivation of NR1–NR2A and NR1–NR2B channels are similar to those described previously in hippocampal neurones (Legendre *et al.* 1993; Vyklický, 1993; Medina *et al.* 1994). Thus, (i) the time constant of the recovery is about 10–60 s; (ii) prolongation of the conditioning stimulation leads to an increase in the time course of recovery; (iii) the maximum degree of NR1–NR2A and NR1–NR2B channel inhibition is about 40%; and (iv) elevation of external Ca^{2+} enhances the degree of NR1–NR2A inhibition and slows down the time of recovery. Our results also did not reveal subunit-specific differences in properties of Ca^{2+}-induced inactivation for NR1–NR2A and NR1–NR2B channels.

This study demonstrates directly that substitution of the native membrane environment of the NMDA channel in neurones for a foreign one does not prevent the Ca^{2+}-induced inactivation and does not change its main properties. This allows us to suggest that the site for this type of Ca^{2+}-induced modulation is either on the NMDA receptor subunit or develops via Ca^{2+}-dependent modulation of interactions between subunits. Our observations do not exclude the possibility of Ca^{2+}-dependent inactivation of NMDA channels via cytoskeleton proteins (Rosenmund & Westbrook, 1993*b*). A number of membrane skeleton proteins are highly conserved in vertebrates and are likely to be present in different cell types (Baines, 1991; Bennett & Gilligan, 1993). Thus, the possibility that the same type of interactions with membrane spanning proteins (in our case the NMDA channel) occur in human kidney cells and in rat hippocampal neurones cannot be excluded. Additional experiments involving modulation of the cytoskeleton of HEK 293 cells expressing NMDA channels need to be conducted.

Two other interesting observations in this study are the progressive decrease of the glutamate-induced current amplitude (run-down) and the acceleration of desensitization in cells expressing the NR1–NR2A channel. These phenomena have been described in nucleated patches from mouse neurones (Sather, Dieudonne, MacDonald & Ascher, 1992) and in freshly isolated (Chizhmakov, Kiskin & Krishtal, 1992) or cultured (MacDonald, Mody & Salter, 1989; Rosenmund & Westbrook, 1993*a*; Vyklický, 1993) hippocampal neurones. They may reflect augmentation of glycine-independent desensitization of the NMDA receptor

(Sather *et al.* 1992) either due to wash-out of intracellular components or through the influx of Ca^{2+} with activation of NMDA channels (Tong & Jahr, 1994).

From the comparative single-channel analysis of NR1–NR2A subunit combinations expressed in oocytes and in HEK 293 cells it has been concluded that 'these widely used expression systems faithfully reproduce many of the properties of native NMDA receptors (Stern, Cik, Colquhoun & Stephenson, 1994). The present study shows two other similarities between recombinant and native NMDA receptors, namely, their transient inhibition by intracellular Ca^{2+}, and the development of run-down and acceleration of desensitization during long-lasting recording. This provides additional possibilities for further investigation of the properties of NMDA receptors and their modulation by second messengers.

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